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Alternative Promoter and 5′ Exon Generate a Novel $G_s\alpha$ mRNA*

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Several species of mRNA have been shown to encode the α subunit of the stimulatory GTP-binding regulatory protein, $G_s\alpha$. The various $G_s\alpha$ mRNAs are generated through alternative splicing of a single precursor RNA and through the use of alternative acceptor splice sites. We now report the existence of a $G_s\alpha$ mRNA that uses a previously unidentified promoter and leading exon (termed exon 1′). In both the canine and human $G_s\alpha$ genes, exon 1′ is located 2.5 kilobases 5′ of exon 1. Exon 1′ does not contribute an in-frame ATG, and thus its mRNA encodes a truncated form of $G_s\alpha$. Initiation of translation is predicted to begin at an AUG in exon 2, as demonstrated both by *in vitro* translation and COS cell expression studies.

G proteins include a family of heterotrimeric guanine nucleotide binding regulatory proteins (α, β, and γ subunits) that couple receptors for light, neurotransmitters, and hormones to a variety of effector mechanisms (1). The α subunit appears to play the principal role in signal transduction, whereas the β and γ subunits appear to be involved in modulating the activity of the signal transduction pathway. The α subunit of the stimulatory G protein, $G_s\alpha$, couples receptors to adenylate cyclase and is encoded by a single, 13-exon gene spanning 20 kb[1] (2). A variety of tissues contain two major forms of $G_s\alpha$, which are encoded by mRNAs generated by alternative splicing of exon 3; inclusion of this exon results in the insertion of 15 amino acid residues (3). Further diversity results from alternative splicing at exon 4, which allows for the generation of two short and two long forms of $G_s\alpha$ that differ only by the addition of a serine residue (2). We now report the existence of a $G_s\alpha$ mRNA that uses a previously unidentified promoter and leading exon and describe the sequence and location of this alternative promoter-exon element.

MATERIALS AND METHODS

Cloning and Sequencing Determination—The λgt10 cDNA library was prepared from canine ventricular RNA according to the method of Watson and Jackson (4). 2×10^6 recombinant colonies were screened with a probe containing 1 kb of the 3′ end of a mouse $G_s\alpha$ cDNA. Prehybridization was carried out for at least 2 h in 50% formamide, 5 × SSC, 5 × Denhardt's, 25 mM $NaPO_4$ (pH 6.5), 0.25 mg/ml calf thymus DNA, and 0.1% SDS at 42 °C. Hybridization was then performed in the same solution (solution A) at 42 °C containing at least 2×10^5 cpm/ml of nick-translated probe for a period of 12 to 16 h followed by washing under increasingly stringent conditions. For genomic DNA cloning, 3×10^6 recombinant clones from an EMBL3 canine genomic DNA library were screened with a probe that contained 170 bases of the novel sequence from a cDNA clone, 6C. The probe was labeled with [^{32}P]-dCTP by the multiprimer random labeling method. The protocol for hybridization was identical with that described above. All DNA sequencing (using the universal or synthetic oligonucleotide primer) was carried out bidirectionally at least twice by the dideoxy chain termination method of Sanger *et al.* (5) after the restriction fragments had been subcloned into M13 vectors.

Primer Extension—10 μg of poly(A)$^+$ RNA from canine ventricle or 10 μg of tRNA as a control was hybridized at 30 °C for 16 h to a $^{32}PO_4$-end-labeled 20-base oligonucleotide in a solution of 50% deionized formamide, 1 mM EDTA, 20 mM PIPES (pH 6.4), and 0.4 M NaCl. Following precipitation, washing, and resuspension in buffer, the hybridized primer was extended with reverse transcriptase at 50 °C for 1 h, and the product was then separated on a 6% polyacrylamide gel followed by autoradiography. The size of each band was determined by using both a sequence ladder and labeled DNA size markers.

S1 Mapping Analysis—50 to 80 μg of whole RNA from canine ventricle or 10 μg of tRNA as a control was hybridized to a 0.3-kb, $^{32}PO_4$-end-labeled *Nar*1-*Nar*1 fragment. Hybridization was carried out in a solution of 80% deionized formamide, 0.1% SDS, 1 mM EDTA, 20 mM PIPES (pH 6.4), and 0.4 M NaCl at 65 °C for 16 h. The hybridized DNA and RNA were then digested with 150 units of S1 nuclease at 14 °C for 30 min. The product was separated on a 6% polyacrylamide gel followed by autoradiography. Labeled DNA size markers and a sequence ladder were used to determine the size of each band.

Northern Blot Analysis—30 to 50 μg of whole RNA were hybridized to a $G_s\alpha$ cDNA probe (exons 1–13), or to a 170-bp probe specific for exon 1′. Hybridization was carried out in solution A for 24 h after prehybridization for 12 h, at 42 °C with a $G_s\alpha$ cDNA probe, and at 60 °C with a 170-bp probe. The blot with a $G_s\alpha$ probe was then washed for 1 h in a solution consisting of 0.2 × SSC and 0.2% SDS at 60 °C. The blot with a 170-bp probe was washed for 1 h in a solution consisting of 0.1 × SSC and 0.2% SDS at 70 °C.

In Vitro Translation and Immunoprecipitation—cDNAs with their own 3′ untranslated regions encoding either the novel (exons 1′ to 13) or the conventional form (exons 1 to 13, without exon 3) of $G_s\alpha$ were cloned into pGEM3 plasmids from whose polylinker the ATG had been excised. Capped mRNAs were synthesized with SP6 RNA polymerase as described by Olate *et al.* (6). The translation of the mRNAs (2 μg of mRNA per 37 μl of lysate in a final volume of 50 μl) was carried out using a reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. Five μl of the reaction mixture was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography for 15 h. Immunoprecipitation of 5 μl of the translated products was carried out according to the method of Jagus (7) with slight modifications, using an antipeptide antiserum (1:200) directed against the COOH terminus of $G_s\alpha$ protein (Cys ^{379}-Leu ^{394}).

Expression in COS Cells—cDNAs were cloned into the modified pMT2 vector (8). COS cells were transfected either with a plasmid containing the novel form of $G_s\alpha$ (exons 1′–13) or the conventional

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[1]The abbreviations used are: kb, kilobase(s); bp, base pair(s); SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazinediethanesulfonic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

form of $G_s\alpha$ (exons 1–13) as described. Seventy-five μg of membrane, prepared 48 h after transfection, was solubilized, separated on SDS-polyacrylamide gel electrophoresis, transferred to Immobilon P membrane, and immunoblotted with the same antiserum as above using a protocol similar to that described by Robishaw *et al.* (3).

RESULTS AND DISCUSSION

From the canine ventricular cDNA library, besides three of four splice variants of $G_s\alpha$ cDNA described previously (9), we also obtained one cDNA, 6B, that diverged near the 5′ end of its translated region (10). We later identified the position of divergence as the boundary between exons 1 and 2, on the basis of the $G_s\alpha$ gene structure reported by Kozasa *et al.* (2). cDNA 6B was similar to typical $G_s\alpha$ cDNA in that it contained sequences contributed by exons 2–13, but differed in the region 5′ of exon 2, where there were 73 bp of unique sequence. We rescreened the library with a 64-base oligonucleotide whose sequence was based on the cDNA 6B region 5′ of exon 2. Even though the longest cDNA we obtained, 6C, extended 183 bp 5′ of the exon 2 junction, we could not identify an in-frame ATG. All cDNAs obtained from the second screening were otherwise identical, except for splice variants either containing or lacking exon 3.

To characterize the exon(s) containing this sequence and not previously identified as part of the $G_s\alpha$ gene, we screened a canine genomic DNA library in EMBL3 with a 170-bp fragment of the sequence unique to cDNA 6C. We found one clone which contained a 14-kb insert that hybridized not only to the unique 170-bp probe but also to probes specific for exons 1 and 2 of $G_s\alpha$. From the restriction map of this insert, we concluded that the novel sequence represented a previously unidentified exon (termed exon 1′) located 2.5 kb 5′ of exon 1 (Fig. 1*A*). We then sequenced the regions flanking this exon in M13. After the 5′ boundary (CAP site) of exon 1′ had been mapped, it was apparent that an in-frame ATG was lacking (Fig. 1*C*).

We performed both primer extension and S1 nuclease mapping studies to determine the transcription initiation site of exon 1′ (Fig. 2). Primer extension study identified a single 120-base extension product, and S1 nuclease mapping showed a 97-base extension product. Both were in agreement in locating the major transcription initiation site at the position indicated in Fig. 1*C*. This position is ~15 bases upstream of the most 5′-extended cDNA originally cloned from our library. However, we later cloned one cDNA which was even further 5′-extended, indicating that other transcription initiation sites are rarely employed.

As has been reported for the putative promoter elements in the 5′ region flanking exon 1 (2), we found no typical "TATA" or "CAAT" box in the region immediately 5′ of exon 1′. Similarly, the "GC" content of the regulatory region of exon 1′ was high (76%). These characteristics, together with evidence of more than one transcription initiation site, are features common to the so-called "housekeeping" genes (11–13). GC boxes (GGGCGG, CCGCCC (14)) were present at positions −415, −381, −295, and −208 of exon 1′. These sequence elements may serve as binding sites for the Sp1 transcription factor, which is thought to be important in regulating transcription of the SV40 early region genes. Similarly, multiple GC boxes are found in the 5′ flanking region of exon 1, as well as in the promoter regions of several housekeeping genes. We also found other putative regulatory elements 5′ of exon 1′, including sequences similar to OCT-1 (CTTTGCAC, position −304) and AP-2 (CCCCCGGC, position −220) binding sites (15).

The sequence of exon 1′ itself also has a high GC content (78%) and an extended (21-bp) palindromic sequence at its

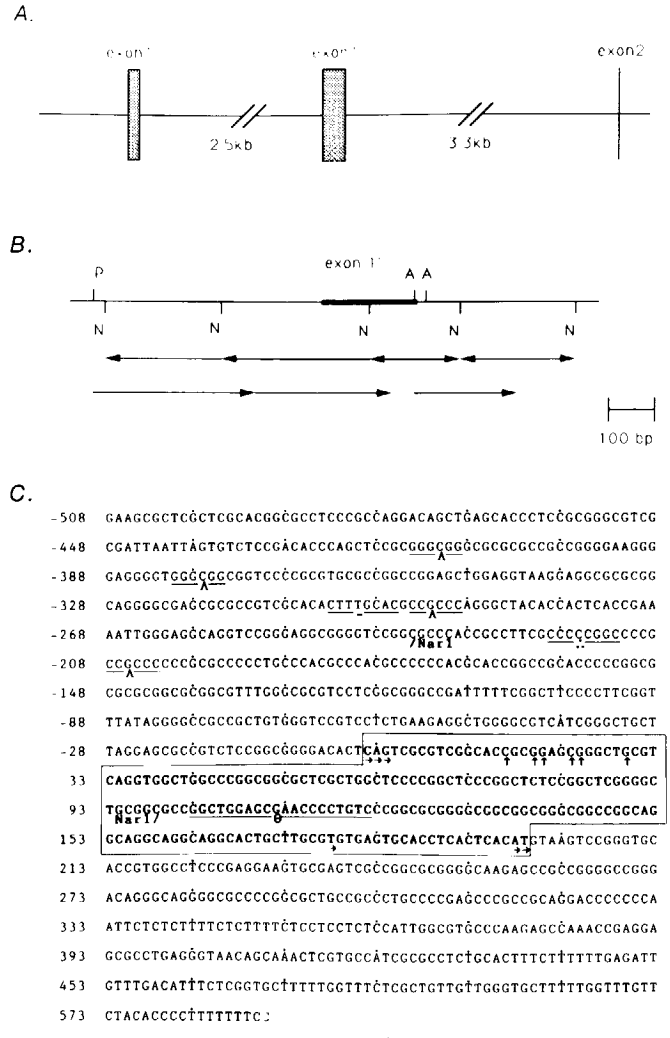

FIG. 1. **Partial structure, restriction map, and sequences of the canine $G_s\alpha$ gene.** *A*, schematic diagram of the upstream portion of the canine $G_s\alpha$ gene. Exons and introns are shown as *boxes* and *lines*. *B*, restriction map and strategy of sequencing. Direction and extension of sequencing are indicated by *arrows*. *N* denotes *Nar*1 site; *A* denotes *Apa*L1 site; *P* denotes *Pst*1 site. *C*, sequence characteristics of exon 1′ and its flanking regions. The putative promoter region and intron are shown, with exon 1′ *boxed*. The symbol ⟶⟶ indicates the major transcription initiation site, the symbol ⟶ indicates the exon-intron splice junction. *Arrows* show 5′ ends of cDNAs obtained from the canine ventricular cDNA library. Λ denotes a GC box and its inverted sequence, ~ denotes the OCT-1 binding element, ∴ denotes the AP-2 binding element, → denotes the beginning of an extended palindromic sequence, and θ denotes the 20-mer used for primer extension.

3′ end, TGTGAGTGCACCTCACTCACA. Similar patterns are characteristic of various hormone response elements; for example, a 13-bp (estrogen) or a 15-bp (glucocorticoid) hormone response element is found in the upstream promoter region of genes regulated by steroid hormones (16). We also identified one cDNA that lacked this particular 21-bp sequence, such that the termination of exon 1′ (splice site to exon 2) occurred 21 bp upstream of the usual donor splice site. A splice junction consensus sequence, T/GTGAG (17), was also present at this position. Although the T/GTGAG sequence may account for this splicing pattern, the palindromic nature of the 21-bp sequence likely confers a secondary structure (stem loop) that might affect RNA precursor processing. A GGCT sequence is repeated eight times at positions 25, 38, 60, 68, 75, 83, 90, and 102 of exon 1′ (Fig. 1*C*); a tandem direct repeat, GGCA/GGCA/GGCA/GGCA/GGCA,

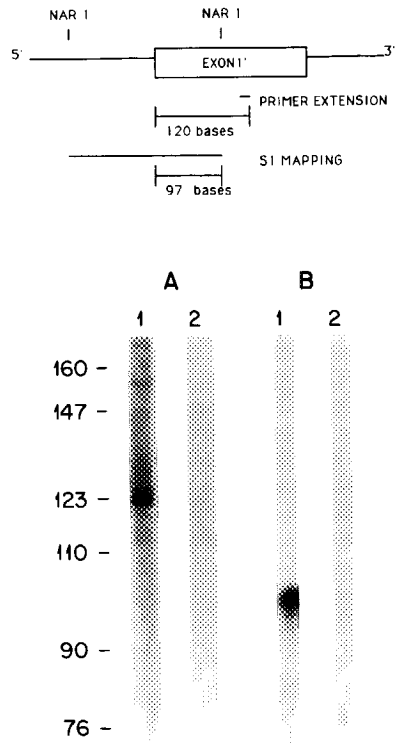

FIG. 2. **Primer extension and S1 nuclease mapping of the transcription start site of the novel form of canine $G_s\alpha$ mRNA.** *A*, primer extension: 10 μg of poly(A)$^+$ RNA from canine ventricle (*A1*) or 10 μg of tRNA (*A2*) as a control was used, as described under "Materials and Methods." A $^{32}PO_4$-end-labeled 20-base oligonucleotide (θ in Fig. 1*C*) was used as a primer. *B*, S1 nuclease mapping: 50 to 80 μg of whole RNA from canine ventricle (*B1*) or 10 μg of tRNA (*B2*) as a control was hybridized to a 0.3-kb, $^{32}PO_4$-end-labeled *Nar*1-*Nar*1 fragment (*cf.* Fig. 1*C*).

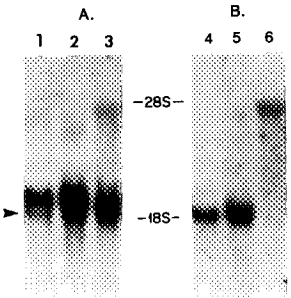

FIG. 3. **Northern blot analysis of canine and human RNA.** *A*, 30 to 50 μg of whole RNA from canine ventricle (*A1*), canine brain (*A2*), and human ventricle (*A3*) were hybridized to a $G_s\alpha$ cDNA probe (exons 1–13) made by the multiprimer random labeling method. The *arrow* points to the minor band that represents mRNA generated by using exon 1'. *B*, 40 to 80 μg of RNAs from canine ventricle (*B4*), canine brain (*B5*), and human ventricle (*B6*) were hybridized to a 170-bp probe specific for exon 1'.

begins at position 148. We do not know whether these regions function as transcriptional or post-transcriptional (RNA) regulatory elements.

We next examined the two forms of $G_s\alpha$ mRNA expressed by alternative transcription of exon 1 or exon 1'. With a ^{32}P-labeled conventional $G_s\alpha$ cDNA probe (exons 1 to 13), the analysis revealed a major and a minor band, differing in size by ~0.3 kb (Fig. 3*A*, *arrow*). The ratio of the major band (known to be $G_s\alpha$ mRNA because it could be detected with an exon 1-specific probe as well) to the minor band was at least 10:1 as determined by densitometric scanning. With a 170-bp probe specific for exon 1', only the minor band was observed. Hybridization to the exon 1' probe was done under highly stringent conditions to avoid any nonspecific labeling. The tissue distribution of the novel (exon 1') $G_s\alpha$ mRNA species was similar to that of the conventional form, although there was less expression of the novel form in the liver.[2] An intriguing, but as yet unexplained, finding was that in human heart a much larger RNA species was detected, both with a conventional $G_s\alpha$ cDNA probe and the exon 1'-specific probe (Fig. 3), again under highly stringent conditions.[3] It is also noteworthy that only the larger mRNA species was detected with the exon 1'-specific probe in the human. The larger mRNA species from human heart most likely represents a mature mRNA that contains exons 1' and 1 (with the intervening sequence between them).

A major question arose as to the nature and function of the putative protein product encoded by the mRNA that uses exon 1' in the dog. It was not clear to us which ATG would be employed for translation since the first in-frame ATG (at amino acid position 60 in exon 2 (2)) is not found within the context of a preferred Kozak consensus sequence (18). In fact, the next ATG in exon 5 (at amino acid position 110) is contained within a sequence that would be expected to be more favorable for translation initiation. Therefore, to determine the efficiency of translation initiation and the size of the major product, we performed *in vitro* translation experiments using a reticulocyte lysate and $G_s\alpha$ mRNA. As can be seen in Fig. 4*A*, major and minor products were generated with either mRNA; however, in each case, the predominant species reflects initiation at the first available in-frame ATG, based on their predicted M_r (6). It is important to note that for any given quantity of mRNA, a greater amount of the

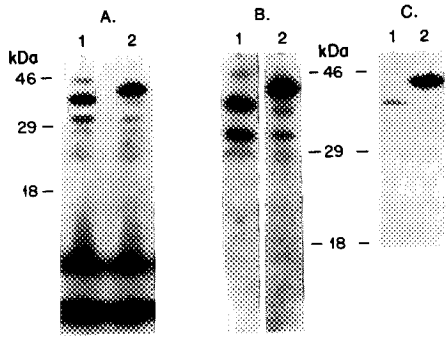

FIG. 4. ***In vitro* and *in vivo* translation of canine $G_s\alpha$.** *A*, *in vitro* translation of canine $G_s\alpha$ mRNAs. cDNAs with their own 3' untranslated regions encoding either the novel (*A1*) or the conventional (*A2*) form of $G_s\alpha$ were used. The translation of the mRNAs was carried out as described under "Materials and Methods." When mRNA was excluded from the reaction mixture, these bands were not observed. The same result was confirmed by at least five independent experiments. *B*, immunoprecipitation of the translated products in *A*, using an antipeptide antiserum (1:200) directed against the COOH terminus of $G_s\alpha$ protein (Cys^{379}-Leu ^{394}) (*B1*, the novel form; *B2*, the conventional form). Irrelevant anti-peptide antibodies employed as negative controls failed to precipitate these proteins. The autoradiographs were exposed for 48 h. *C*, expression in COS cells: COS cells were transfected either with a plasmid containing the novel form of $G_s\alpha$ (exons 1'–13, *C1*) or the conventional form of $G_s\alpha$ (exons 1–13, *C2*) as described. Irrelevant anti-peptide antibodies employed as negative controls failed to detect these bands. In cyc$^-$ membranes, no labeling was detected with this antiserum. The same result was obtained in two separate transfections. The autoradiographs were exposed for 3 h.

[2]These results were also confirmed by S1 nuclease analysis using a single-stranded end-labeled cDNA probe that spanned the exon 1'-exon 2 junction.

[3]Canine exon 1' is highly homologous (90%) to human exon 1' (unpublished data).

conventional $G_s\alpha$ product was synthesized, possibly reflecting the presence of a preferred Kozak consensus sequence (18) in $G_s\alpha$ mRNA. The ~38-kDa product translated from the novel form of $G_s\alpha$ mRNA is of the appropriate size in view of the amino-terminal deletion of 59 amino acids and the addition of 15 amino acids contributed by exon 3, which was present in the cDNA template. A COOH terminal-directed anti-peptide $G_s\alpha$ antibody was capable of immunoprecipitating the major products translated from either mRNA species (Fig. 4*B* and below).

We also examined translation of the conventional and novel forms of $G_s\alpha$ mRNA (with the 15 amino acids from exon 3) in COS cells by using a plasmid construct containing SV40 origin of replication and promoter elements (19). Cells were transfected with a plasmid containing either the long form of $G_s\alpha$ (exons 1–13) or the novel form of $G_s\alpha$ (exons 1′–13). As can be seen in Fig. 4*C*, a discrete band at M_r = ~38,000 is present in the lane loaded with membranes solubilized from cells transfected with the novel form of $G_s\alpha$ cDNA. The amino acid position for initiation of translation identified by the COS cell studies recapitulates the findings obtained by *in vitro* translation. We also examined a cytosolic preparation of the COS cells to determine whether the truncated isoform may simply not have localized to the membrane fraction because of the deletion of the amino terminus. It has been postulated that an amino-terminal domain of the α subunit is responsible for membrane attachment, possibly through an association with $\beta\gamma$ subunits (20). However, the truncated isoform was not detectable in the cytosolic fraction by Western blotting.

On the basis of the homology of $G_s\alpha$ with other GTP-binding regulatory proteins and the x-ray crystallographic structures of two related proteins, $p21^{ras}$ and elongation factor Tu, it has been proposed that amino acid positions 40–60 in $G_s\alpha$, encoded by exons 1 and 2, constitute a functional domain that contributes to GTP binding and GTPase activity (21). Recently, studies from the laboratories of Bourne and Gilman and co-workers (22, 23) have demonstrated that mutations within this site alter the ability of the mutant proteins to hydrolyze GTP and to interact with adenylate cyclase. Therefore, we examined whether the amino-terminal-truncated protein encoded by the novel form of $G_s\alpha$ mRNA (~38-kDa species) maintained any of the functional properties characteristic of $G_s\alpha$. Since the ~38-kDa species contained the primary sequence known to undergo ADP-ribosylation by cholera toxin, we assessed whether the product translated *in vitro* or the protein expressed in COS cells could serve as a substrate for cholera toxin labeling in the presence of [^{32}P]-NAD (24). Cyc^- membranes were included to serve as a source of ADP-ribosylation factor. However, no labeling attributable to the ~38-kDa species could be detected. This finding suggests that the conformation required to serve as a cholera toxin substrate may not have been maintained in the novel form of $G_s\alpha$. Similarly, the protein prepared by either method could not reconstitute NaF-stimulated adenylate cyclase activity in cyc^- membranes (25). As a positive control, we did demonstrate this activity with $G_s\alpha$ expressed in COS cells and, to a lesser extent, with $G_s\alpha$ produced by *in vitro* translation. However, the fact that the amount of protein generated by the novel form of $G_s\alpha$ mRNA was severalfold less than that generated by the conventional form may complicate interpretation of these findings (*cf.* Fig. 4*C*). This difference could reflect the presence of a preferred consensus sequence for translation initiation in the conventional form (18).

Finally, we attempted to determine whether the ~38-kDa $G_s\alpha$ protein could bind GTP by direct assay with [^{35}S]GTPγS, by ligand blotting as described for certain GTP binding proteins by McGrath *et al.* (26), and by direct photo-cross-linking with [^{32}P]GTP (27). Although we could not detect GTP binding to the ~38-kDa protein, we cannot exclude the possibility that it does indeed bind GTP because the amount of protein available for assay may have been a limiting factor. Such a conclusion must await expression of the pure protein in a quantity sufficient to allow direct binding assays.

Our study shows that the cell can use alternative promoters in transcribing the canine $G_s\alpha$ gene. This previously unknown mechanism adds, in conjunction with alternative splicing of exon 3, to the array of mRNA isoforms that can be generated from this single gene. Exon 1′ and its 5′ flanking region are also found in the human $G_s\alpha$ gene; the sequences are 90% homologous between the two species.[3] It will be important to determine the patterns of expression of mRNAs that incorporate exon 1′ in a variety of species, as well as the patterns of expression during development. Although the use of alternative promoters is observed with a variety of other genes, it is noteworthy that the use of exon 1 *versus* exon 1′ in the $G_s\alpha$ gene does not appear to be regulated in a tissue-specific manner (based on Northern blot analysis of RNA from multiple canine tissues). It has been recognized that alternative start sites for transcription allow two forms of a protein to be translated from a single gene (28). In the case of $G_s\alpha$, the AUG encoded by exon 1 lies within a favorable context for translation initiation, precluding utilization of potential downstream start sites. However, the generation of a second form of mRNA from a leading exon which lacks an in-frame ATG allows a shortened form of the protein to be produced by translation initiation from a downstream AUG. In the case of the $G_s\alpha$ gene, this AUG lies within exon 2. Other genes use this mechanism to generate protein products that have different biological functions or are targeted to different locations within the cell (28).

The COS cell expression studies suggest that, despite the lack of the 59 amino-terminal residues, the truncated protein still localizes to the membrane. However, its level of expression in the COS cell was much lower than that of the conventional form of $G_s\alpha$. It may be that low steady state levels of the truncated isoform are a reflection of both the lower levels of mRNA encoding the isoform and the lack of an optimal consensus sequence at the AUG employed for translation initiation. Although we have not yet identified an activity that the truncated isoform shares with $G_s\alpha$, it is possible that discrete domains within this putative protein product maintain functional integrity.

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